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Purification of *Pseudomonas putida* Acyl Coenzyme A Ligase Active with a Range of Aliphatic and Aromatic Substrates

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Acyl coenzyme A (acyl-CoA) ligase (acyl-CoA synthetase [ACoAS]) from *Pseudomonas putida* U was purified to homogeneity (252-fold) after this bacterium was grown in a chemically defined medium containing octanoic acid as the sole carbon source. The enzyme, which has a mass of 67 kDa, showed maximal activity at 40°C in 10 mM K_2PO_4H-$NaPO_4H_2$ buffer (pH 7.0) containing 20% (wt/vol) glycerol. Under these conditions, ACoAS showed hyperbolic behavior against acetate, CoA, and ATP; the K_ms calculated for these substrates were 4.0, 0.7, and 5.2 mM, respectively. Acyl-CoA ligase recognizes several aliphatic molecules (acetic, propionic, butyric, valeric, hexanoic, heptanoic, and octanoic acids) as substrates, as well as some aromatic compounds (phenylacetic and phenoxyacetic acids). The broad substrate specificity of ACoAS from *P. putida* was confirmed by coupling it with acyl-CoA:6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum* to study the formation of several penicillins.

Pseudomonas species and other related bacteria can degrade a wide variety of carbon sources through different catabolic pathways (3, 4, 7–10, 12–14, 16, 17, 34, 36, 37).

We have recently reported that *Pseudomonas putida* U is able to grow efficiently in a chemically defined medium containing phenylacetic acid (PAA) as the sole carbon source (33). In this case, the degradation of PAA seems to be related to the induction of an enzyme which activates this aromatic compound to phenylacetyl-coenzyme A (PA-CoA). This reaction is catalyzed by a newly described enzyme (phenylacetyl-CoA ligase [EC 6.2.1-]; PCL) (33) which is specifically induced by PAA and which is under genetic control by carbon catabolite repression (30). Study of the substrate specificity of this enzyme by different methods—high-pressure liquid chromatography (HPLC; 33), spectrophotometry and fluorometry (35), colorimetry (33), or bioassay against *Micrococcus luteus* (after coupling PCL and acyl-CoA:6-aminopenicillanic acid [6-APA] acyltransferase [AT] from *Penicillium chrysogenum*; 32)—has shown that this protein can preferentially recognize PAA, although many other aromatic and aliphatic compounds can also be activated to their acyl-CoA derivatives (31). To establish the differences between PCL and other acyl-CoA-activating enzymes, we studied a different acyl-CoA synthetase (ACoAS) obtained after *P. putida* was grown in the same chemically defined medium in which PAA was replaced by octanoic acid, with this molecule as the sole carbon source. A comparative study of this enzyme with other ACoASs previously described, as well as of its utilization for the enzymatic synthesis of antibiotic substances, is made, and the results are discussed.

MATERIALS AND METHODS

Materials. ATP, CoA, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, reduced glutathione, 2-mercaptoethanol, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, and 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were supplied by Sigma Chemical Co. Δ^2-Pentenylpenicillin (F; 1,510 U/mg), *n*-amylpenicillin (DF; 1,625 U/mg), *n*-hexylpenicillin (1,540 U/mg), *n*-heptylpenicillin (K; 2,400 U/mg), Δ^2-heptenylpenicillin (1,760 U/mg), benzylpenicillin (G; 1,590 U/mg), phenoxyacetylpenicillin (1,540 U/mg), and 6-aminopenicillanic acid either were gifts from Antibioticos S.A. (León, Spain) or were synthesized by us. Protein molecular weight standards, Sephadex G-25 (PD-10), DEAE-Sephacel, Sephacryl S-200 Superfine, Phenyl-Sepharose CL-4B, and Sepharose CL-6B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other products were of analytical quality or HPLC grade.

Enzymes. PCL from *P. putida* and AT from *P. chrysogenum* were purified as reported elsewhere (1, 33).

Microorganisms. The *P. putida* (strain U) used in the experimental work was from our collection. The strain was originally obtained from R. A. Cooper (Department of Biochemistry, University of Leicester, Leicester, United Kingdom).

M. luteus ATCC 9341 was used for determination of the different penicillins by bioassay (28).

The strains were kept lyophilized.

Culture media and growth conditions. *P. putida* was maintained and cultured as described elsewhere (33). The medium used for the growth was a chemically defined one with the following composition (in grams per liter): KH_2PO_4 (13.6), $(NH_4)_2SO_4$ (2.0), $MgSO_4 \cdot 7H_2O$ (0.25), $FeSO_4 \cdot 7H_2O$ (0.0005), biotin, (0.001), and octanoic acid (2.8). The pH of the medium was adjusted to 7.0 with KOH (30% [wt/vol]). When necessary, the carbon source was replaced by other acids (acetic, butyric, and hexanoic acids) at the same final concentration (2.8 g · liter^{-1}). Incubations were carried out at 250 rpm and 30°C.

Acyl-CoA ligase assay. Acyl-CoA ligase activity was monitored by measuring the rate of formation of acetylhydroxamate in the presence of ATP, CoA, acetic acid, and neutral hydroxylamine as described for other CoA-activating enzymes (22). We used acetate instead of other acids longer than C_6 (hexanoic, heptanoic, and octanoic acids), because these compounds do not generate a colored product or do so

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TABLE 1. Purification of ACoAS of *P. putida* U

Treatment	Vol (ml)	Amt of protein (mg)	Enzyme activity (U)	Sp act (U/mg of protein)	Yield of recovery (%)	Purification (fold)
Crude extract	160	1,536	3,280	2.13	100	1
Ammonium sulfate precipitation (20–45%)	80	456	3,190	6.99	97.3	3.28
Ultracentrifugation (250,000 × *g*, 45 min)	69	376	2,889	7.68	88.08	3.61
Phenyl-Sepharose (fractions 100–114)	33.30	12	1,010	84.16	30.79	39.51
DEAE-Sephacel eluate (gradient 0.12–0.17 KCl; fractions 32–42 concentrated)	20.35	8.25	925	112.12	28.20	52.64
Sephacryl S-200 eluate (fractions 44–54 concentrated)	23.65	1.74	770	442.27	23.47	207.64
Sepharose CL-6B eluate (fraction 85)	2.2	0.16	86	537.50	2.62	252.35

at a very low rate when incubated with hydroxylamine (18). For this reason, a different assay method (bioassay against *M. luteus*; see below) was used to study the substrate specificity of this enzyme (see below).

The reaction mixture contained $MgCl_2$ (0.2 M; 12.5 μl), ATP (0.1 M; 50 μl), CoA (20 mM; 30 μl), sodium acetate (0.2 M; 30 μl), and hydroxylamine solution (prepared as described below; 50 μl). All substrates except $MgCl_2$ (water) were dissolved in 10 mM K_2HPO_4-NaH_2PO_4 buffer (pH 7.0) containing 20% (wt/vol) glycerol. When required, acetic acid was replaced by other acids. In standard tests, sodium acetate, ATP, $MgCl_2$, or CoA was omitted. After 5 min of temperature equilibration in a water bath at 37°C (or at the temperature assayed), 100 μl of enzyme solution was added, and the incubations were carried out for 20 min or the required time. The reactions were stopped by adding 450 μl of the ferric-chloride reagent (see below), and the mixtures were kept on ice for 30 min. At this time, the tubes were centrifuged in an Eppendorf 5414 Microfuge for 2 min, and the red-purple color generated was measured at 540 nm with a Shimadzu UV-120-02 spectrophotometer. The extinction coefficient of acetylhydroxamate under these conditions was 0.975 mM^{-1} cm^{-1}. One unit of enzyme activity is defined as the catalytic activity leading to the formation of 1 nmol of acetylhydroxamate in 1 min. Specific activity is given as units per milligram of protein.

The amount of protein was measured by the method described by Bradford (5), with bovine serum albumin as the standard.

Neutral hydroxylamine solution and the ferric-chloride reagent were prepared as reported elsewhere (27, 33).

Purification of acyl-CoA ligase from *P. putida*. Bacteria grown in the medium described above for 22 h were harvested by centrifugation (10,000 × *g* for 10 min at 2°C), washed twice with sterile saline solution, and resuspended in 500 mM K_2PO_4H-$NaPO_4H_2$ buffer to pH 7.1 (P buffer) containing 5 mM mercaptoethanol, 4 mM EDTA, and 1 mM PMSF. Cells were disrupted with glass beads (Ballotini; 0.17 to 0.18 mm in diameter) with a Braun MSK mechanical disintegrator. Cell debris was eliminated by centrifugation at 17,000 × *g* for 10 min at 2°C. The pellet was discarded, and the supernatant fluid (160 ml) was precipitated with ammonium sulfate. The fraction precipitating between 20 and 45% (containing all of the acyl-CoA ligase activity, evaluated with acetate as the substrate) was collected, dissolved in P buffer (80 ml), and precipitated again with ammonium sulfate (30% saturation). The precipitate was resuspended in the same buffer containing ammonium sulfate (15% saturation) and was ultracentrifuged (250,000 × *g* for 45 min).

The precipitate was eliminated, and the supernatant containing the whole enzyme was used for the following steps. The ultracentrifuged extract was applied to a Phenyl-Sepharose CL-4B column (2.0 by 18 cm) previously equilibrated with P buffer containing ammonium sulfate (20% saturation). The column was washed with 130 ml of the same buffer and later with 90 ml of P buffer without salt. The ACoAS activity was eluted with a glycerol gradient (0 to 16% [wt/vol]) (aliquots, 2.2 ml each). ACoAS activity eluted between tubes 100 and 120, showing a peak in tube 106. Fractions 100 to 114 were combined and precipitated with ammonium sulfate (65% saturation). The precipitate was dissolved in 10 mM K_2PO_4H-$NaPO_4H_2$ buffer (pH 7.15) containing 5 mM mercaptoethanol, 4 mM EDTA, and 5 mM $MgCl_2$ and 20% (wt/vol) glycerol (PG buffer), and the mixture was applied to a Sephadex G-25 PD column equilibrated with the same buffer. Aliquots (three to six) containing ACoAS activity were applied to a DEAE-Sephacel column (1.7 by 6.5 cm). The column was washed with 52 ml of PG buffer and eluted with a KCl gradient (0.12 to 0.17 M) (aliquots, 1.9 ml each). ACoAS eluted between tubes 32 and 44, with a maximum in tubes 34 to 37. Fractions 32 to 42 were mixed and precipitated with ammonium sulfate (65% saturation). The precipitate was dissolved in 1 ml of PG buffer and applied to a Sephacryl S-200 column (2.5 by 34 cm). Aliquots of 2.15 ml each were collected (flow rate, 15 ml/h) and assayed. ACoAS eluted between tubes 43 and 57, with a peak of activity in fractions 47 and 48. Fractions 44 to 54 were combined, precipitated with ammonium sulfate (65% saturation), dissolved in 1 ml of buffer identical to PG but without glycerol, and applied to a Sepharose CL-6B column (3 by 42 cm). Aliquots of 2.2 ml each were collected (flow rate, 20 ml/h) and assayed. ACoAS eluted between fractions 78 and 92, showing a peak of activity in fraction 85 (160 μg of protein). By this procedure, the enzyme was purified to homogeneity 252-fold (Table 1).

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (sodium dodecyl sulfate [SDS]-PAGE) was performed in 10% slab gels (26) with phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa) as molecular mass standards.

Determination of molecular mass of acyl-CoA ligase from *P. putida* U. Molecular masses were determined by using a calibrated Sephacryl S-300 column with the following known proteins: apoferritin (433 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (29 kDa).

Acyl-CoA ligase (ACoAS) and acyl-CoA:6-APA AT coupled assay. The coupled ACoAS-AT enzymatic system (see Fig. 1) contained the following: $MgCl_2$ (0.2 M; 12.5 μl), ATP (0.1

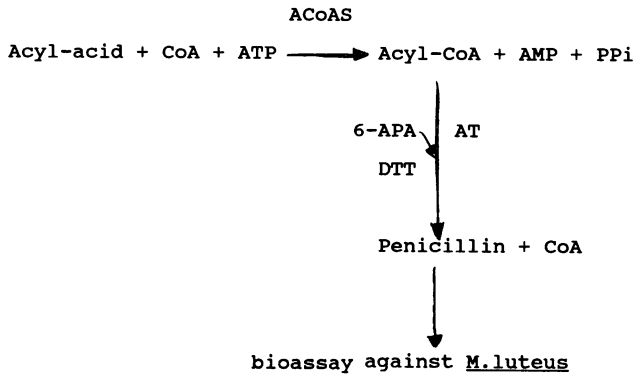

FIG. 1. Sequence of the enzymatic reactions coupled in vitro. DTT, dithiothreitol.

M; 50 μl), CoA (20 mM; 30 μl), penicillin-side chain precursor (acyl or aromatic acids [0.2 M; 30 μl]), 6-APA (0.3 mM; 30 μl [at this concentration, 6-APA does not inhibit the growth of *M. luteus*]), dithiothreitol (20 mM; 10 μl), ACoAS (100 μl; 8 μg of protein), and AT (100 μl; 10 μg of protein). All of the substrates except $MgCl_2$ were dissolved in 50 mM Tris and adjusted to pH 8.0. Incubations were carried out at 30°C for 1 h (or the required time) and halted by the addition of a similar volume of methanol. Control reactions were carried out under the same conditions without penicillin-side chain precursor or CoA. The sequence of the enzymatic reactions coupled in vitro is shown in Fig. 1.

The antibiotics generated were measured by bioassay against *M. luteus* ATCC 9341 as reported previously (32). Each reaction product was evaluated against its corresponding penicillin to facilitate the comparative studies.

RESULTS AND DISCUSSION

P. putida U is able to grow efficiently in a chemically defined medium containing PAA as the sole carbon source (33). Under these conditions, *P. putida* induces a phenylacetate-CoA ligase (EC 6.2.1.-) responsible for the activation of this aromatic compound to phenylacetyl-CoA in the presence of ATP, Mg^{2+}, and CoA. We have purified this protein, and its optimal physicochemical-assay conditions, substrate specificity, and molecular properties have been established (30–33, 35).

To study the characteristics of other analogous enzymes (acyl-CoA-activating enzymes) that can be induced by *P. putida* when this bacterium grows in the presence of different carbon sources, we tested the ability of this strain to grow in the same chemically defined medium containing acetate, butyrate, hexanoate, or octanoate in the place of PAA. The highest growth was obtained when octanoic acid was used as the carbon source, whereas the lowest one was observed in the medium containing acetate (Fig. 2 and 3).

In view of these results, we selected the octanoate-containing medium to study the appearance of ACoAS(s) that should be induced in this bacterium for the degradation of this aliphatic acid.

When *P. putida* U was grown in the octanoate-containing medium, an acyl-CoA ligase activity began to be synthesized at the early logarithmic phase of growth (before 12 h). It increased linearly during the exponential phase, reaching the maximal level at about 22 h; later, between 23 and 30 h, the activity decreased continuously (Fig. 2). For this reason, we

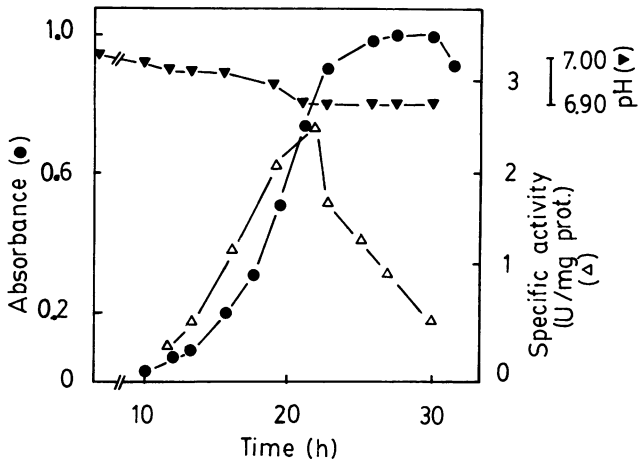

FIG. 2. Time course of ACoAS formation (△), bacterial growth measured as A_{540} (●), and pH variation (▼) when *P. putida* was cultured in a chemically defined medium containing octanoic acid as the sole carbon source. The values of absorbance correspond to those obtained when the bacterial culture was diluted 1/10 with sterile distilled water.

decided to take bacterial cells grown for 20 to 22 h in the medium described above as the source for obtaining and purifying ACoAS.

Physicochemical properties of ACoAS. Purified acyl-CoA ligase from *P. putida* U (see Materials and Methods) runs in SDS–10% PAGE as a single band corresponding to a mass of 67 kDa (Fig. 4). This molecular mass was confirmed by gel filtration with a Sephacryl S-300 column calibrated with several proteins with known molecular masses. By this procedure, we estimated a mass of 65 kDa for ACoAS. These results suggest that in its native form, the enzyme is a monomer. A similar molecular mass has been reported for the subunits of the ACoAS of *Escherichia coli* (24) and acetyl-CoA synthetases isolated from *Methanothrix soehn-*

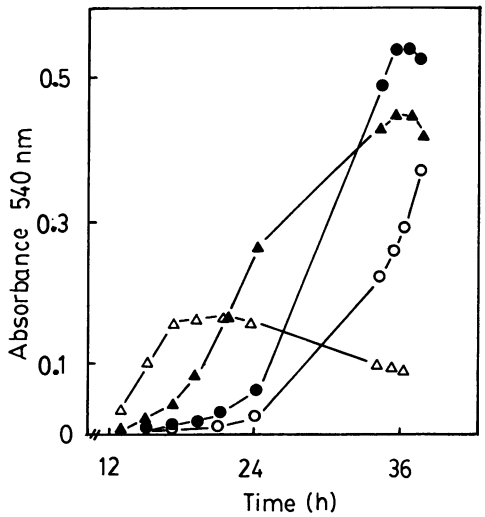

FIG. 3. Growth of *P. putida* in a chemically defined medium containing acetic acid (△), butyric acid (○), hexanoic acid (●), or PAA (▲) as the sole carbon source. The values of absorbance were measured as indicated in the legend to Fig. 2.

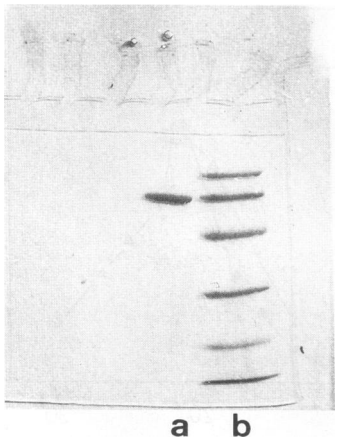

FIG. 4. Electrophoretic mobilities of purified ACoAS from *P. putida* in SDS-10% PAGE (a); proteins used as molecular mass standards (b): α-lactalbumin (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase *b* (94 kDa).

genii (23), baker's yeast (15), and *P. chrysogenum* (29), as well as for the acetyl-CoA ligase of *Neurospora crassa* and *Aspergillus nidulans* (11).

ACoAS activity was maximal at 40°C (Fig. 5). However, at this temperature the enzyme is less stable than at 37°C (data not shown). Accordingly, we used this latter temperature value in the routine assay. Similar optimal temperature values have been reported for the acetyl-CoA synthetases purified from rat liver (38°C), *P. chrysogenum* (37°C), and *M. soehngenii* (35°C), as well as for the ACoASs from *Pseudomonas fragi* (37°C) and *E. coli* (35°C). However, in other cases, the optimal assay temperature was considerably lower (2, 15, 16, 20, 21, 23, 25, 29).

Furthermore, ACoAS showed a maximal rate of catalysis at pH 7.0, although a good activity was also detected at a higher pH value (pH 8.0) with the different buffers employed (Fig. 5). Under the reaction conditions described above, the enzyme shows hyperbolic behavior for ATP, CoA, and acetate; the K_m values calculated for each are 5.2, 0.7, and 4.0 mM, respectively. All of these experiments were carried out at saturating concentrations of Mg^{2+}. This ion can be replaced by Mn^{2+}, but in this case, as has been reported for other analogous enzymes (16, 33), a lower rate of catalysis was observed (about 58%).

Effect of cations and different molecules. To further characterize this enzyme, the effect of several cations on ACoAS activity was studied by adding them to the reaction mixture at a final concentration of 5 mM. In this kind of assay, 50 mM HCl-Tris buffer (pH 7.5) was routinely employed. Monovalent cations (NH_4^+, K^+, Na^+, Li^+) did not cause any significant effect (<5% of both stimulation and inhibition), whereas some divalent cations (Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}) inhibited the enzyme to very different extents (16, 23, 81, 91, and 100%, respectively). ACoAS was also inhibited by several molecules that react with thiol groups (DTNB, *p*-hydroxymercuribenzoate, and *N*-ethylmaleimide) when they were added to the reaction mixture at a concentration of 1 mM (100, 98, and 96%, respectively). These results suggest that in ACoAS from *P. putida*, as in other acyl-CoA-activating enzymes, some SH groups are essential for catalysis (28, 33). The addition of dithiothreitol, reduced glutathione, or 2-mercaptoethanol (5 mM) to the assay did not modify the enzymatic activity.

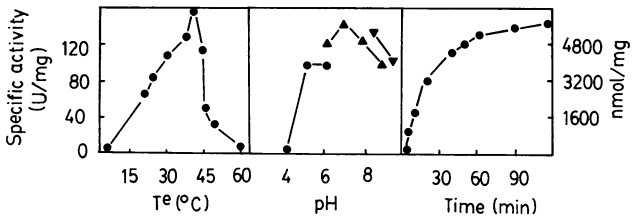

FIG. 5. Effects of temperature (T°), pH (●, 10 mM acetic-acetate buffer; ▲, 10 mM phosphate buffer; ▼, 10 mM glycine-NaOH), and time on the ACoAS activity.

TABLE 2. Comparative study of acyl-CoA ligase and PCL from *P. putida* U

Enzyme	Molecular mass (kDa)	Optimal pH	Optimal temp (°C)	Substrate specificity	
				Hydroxamate procedure[a]	Bioassay[b]
Acyl-CoA ligase	67 (monomer)	7.0	40	C_2–C_6	C_6–C_8,PAA,POA
PCL	48 (monomer)	8.2	30	C_2–C_5, PAA	C_6–C_8,PAA,POA

[a] Evaluation of the enzymatic activity by the hydroxamate procedure. C_2 to C_8 indicates aliphatic monocarboxylic acid, whose carbon length ranges between two and eight carbon atoms. In this group are also included 3-hexenoic and 3-octenoic acids. Acyl-CoA ligase showed maximal activity with acetic acid (100%), and although propionic, butyric, valeric, hexenoic, and 3-hexenoic acids were used as substrates, the activity was lower (27, 34, 62, 58, and 53%, respectively). However, PCL recognizes PAA more efficiently (100%), whereas other molecules, such as acetic, propionic, butyric, and valeric acids, were worse substrates (80, 40, 20, and 10%, respectively).

[b] Evaluation of enzymatic activity by bioassay after both enzymes were coupled with AT from *P. chrysogenum*. With the system acyl-CoA ligase plus AT, higher titers of penicillin were obtained with hexanoic and octanoic acids (1.4 and 1.25 U of penicillin, respectively), whereas when PAA was used as the substrate, five or six times less antibiotic was detected. However, with the system PCL plus AT, the titer of penicillin was maximal when PAA was used as the substrate (5 U), and the lowest titers were with octanoate and 3-octenoate (about 50 to 55 times less).

Substrate specificity. The substrate specificity of ACoAS was studied by measuring the rate of catalysis in the presence of different acids. To evaluate these results, two different methods of assay were employed: a colorimetric one (33) and a biological one (32).

With the first assay (colorimetry), we tested the substrate specificity of ACoAS by evaluating the formation of the different acylhydroxamates when the enzyme was incubated with aliphatic acids ranging between C_2 and C_8. By contrast with the second assay (bioassay against *M. luteus* by coupling ACoAS from *P. putida* and acyl-CoA:6-APA AT from *P. chrysogenum*), we tested several substrates, aromatic or aliphatic, whose carbon lengths range between C_6 and C_8 (or equivalent rigid chain) and which can be transformed into penicillins by AT if they are previously activated to CoA derivatives (32).

Using the colorimetric procedure, we observed that ACoAS from *P. putida* recognized as substrates acetic, butyric, valeric, hexanoic, and 3-hexenoic acids to different extents (Table 2). Other acids with longer acyl chains (octanoic and 3-octenoic acids) could not be assayed by this method, since their corresponding acylhydroxamates are not colored products or they are very slowly formed (18).

However, when the biological assay was employed, we demonstrated that hexanoic, 3-hexenoic, heptanoic, and octanoic acids, as well as some aromatic compounds (PAA and phenoxyacetic acid), are efficiently transformed into the corresponding penicillin (Table 2). These results indicate that ACoAS was able to activate these substrates to their CoA derivatives since, if this were not so, they could not have been used by AT in the coupled enzymatic system (see Materials and Methods).

The comparative study of the substrate specificity of this enzyme with PCL from *P. putida* U indicates that this enzyme is rather different from PCL. Furthermore, it can be concluded that *P. putida* U employs two different enzymes (PCL and ACoAS) for the aerobic catabolism of PAA and octanoic acid, even though both proteins are able to recognize the natural substrate of the other one (ACoAS→PAA and PCL→octanoic acid) (Table 2). The fact that this bacterium was able to induce two acyl-activating enzymes, both having broad and different substrate specificities as a function of the inducer molecule (compound used as carbon source, PAA, or octanoic acid), could be at least one of the reasons to justify the high catabolic potential of this microbe.

The establishment of the molecular bases of this catabolic behavior could be approached by cloning of the gene(s) which codes for these proteins (PCL and ACoAS) as well as for other analogous enzymes (acyl-CoA-activating enzymes), since knowledge of their sequences could help to understand whether these enzymes correspond to either a common or a genetically unrelated family of proteins.

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